The Kinetics of the Reaction of Thiocyanate and Nitrite Ions with *Orconectes limosus* Oxyhemocyanin

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The time course of the reaction of oxyhemocyanin from the arthropod *Orconectes limosus* with SCN^- and NO_2^- was investigated. After addition of one of these anions to oxyhemocyanin solution an exponential decay of A_{340} was found, indicating a removal of oxygen from the active site. The order of reaction with respect to ligand was about 3 for thiocyanate and about 1 for nitrite. On this basis, a possible arrangement of ligands in the active site has been proposed. The final product of reaction with SCN^- yielded a mononuclear copper EPR spectrum while the reaction product with NO_2^- gave a characteristic spectrum of two coupled Cu^{2+} ions. It was possible to remove the ligands from the active site by dialysis. The reaction patterns showed distinct changes in a narrow pH range, 5.3−6.5. A possible effect of structural alterations of the protein moiety on the course of the reaction of oxyhemocyanin with ligands is suggested.

Introduction

Hemocyanins are the proteins transporting oxygen in many arthropod and mollusc species. One oxygen molecule, in the form of the peroxide anion O_2^{2-} is bound to the active site of oxyhemocyanin containing two cupric ions. An intense absorption band at 340 nm, characteristic of the oxy form only, results from an $O_2^{2-} \rightarrow Cu^{2+}$ charge transfer transition [1].

The addition of various compounds to oxyhemocyanin solution results in very distinct changes of the spectroscopic and magnetic properties of the protein [2−4]. So called "spectral derivatives" obtained by various methods provide significant information for the elucidation of the structure and properties of the active site. It has been reported that apart from variations in the structure of the protein part of molluscan and arthropodan hemocyanin, the active sites of these proteins also show considerable differences. Lontie *et al.* [2] demonstrated that fluorides and azides did not react with oxyhemocyanin of the arthropod *Limulus polyphemus* while these agents did react with oxyhemocyanin of the mellusc *Helix pomatia* at a pH dependent rate. Solomon *et al.* [4] observed a reaction with azide and thiocyanate ions for several marine arthropodan hemocyanins.

Thiourea and thiocyanate brought about a fast deoxygenation (in alkaline medium) of molluscan hemocyanin [5]. In EPR spectroscopy, nitrite ions produced a characteristic binuclear signal of a coupled cupric pair and a mononuclear signal [3, 6, 7].

In this study, the interaction of *Orconectes limosus* (a crayfish) oxyhemocyanin with thiocyanate and nitrite at different pH values was investigated.

Materials and Methods

Hemocyanin was isolated from the supernatant of clotted hemolymph by ultracentrifugation ($150\,000 \times g$, 4.5 h). During ultracentrifugation the sedimentation of hemocyanin molecules was accompanied by the sedimentation of high-molecular weight lipid impurities, of dark orange colour. For purification, the sediment was dissolved and applied to a $3.5\,cm \times 50\,cm$ AcA 22 Ultrogel (LKB, France) column and eluted with Tris-HCl (0.1 M, pH 7.6) buffer. Protein elution was monitored by absorbance at 280 nm and 340 nm in a flow − through cuvette. The proper fractions were combined and dialysed against 0.1 M phosphate buffer of desired pH. For optical and EPR measurements, hemocyanin solutions at copper concentrations of about 0.4 mM and 3 mM, respectively, were employed.

To oxyhemocyanin solutions at various pH values NO_2^- or SCN^- were added (in the form of sodium and ammonium salt, respectively) in amounts 10−700 fold higher than the copper content of the

solution. Absorbance at 340 nm was measured as a function of time and of ligand concentration at room temperature.

Copper concentration was estimated by atomic absorption spectroscopy on an AAS 1 (Carl Zeiss Jena, GDR) apparatus. Spectroscopic measurements were made on a SP 1700 (Pye Unicam, England) spectrophotometer and measurements of pH with a Beckman Model 4500 (Beckman, USA) pH meter. EPR spectra were taken at $-150\ °C$ on a JES-ME (Jeol, Japan) spectrometer. A solution of $CuSO_4$ in 25% glycerol in water was used as a standard of mononuclear Cu^{2+} spectrum.

Results

Addition of a several tenfold excess of thiocyanate and nitrite to oxyhemocyanin of *Orconectes limosus* results in a change of the blue colour of the solution at a rate dependent on the type of ligand, its amount and of the pH of the solution. At the same time an EPR signal appears. It demonstrates significant alterations in the structure of the active site of the diamagnetic oxyhemocyanin.

Fig. 1 shows the disappearance of the absorbance at 340 nm at pH 6.1 after addition of a 200-fold excess of SCN^- (with respect to the protein copper). This excess of thio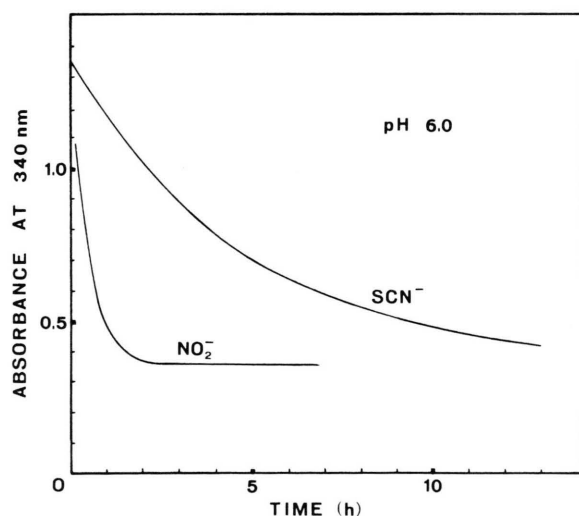cyanate resulted in complete disappearance of the oxy form while a few percent of the oxy form remained at lower concentrations of the ligand. The curve is well approximated by an exponential function indicating a pseudo first-order reaction with respect to oxyhemocyanin. An exponential decay of A_{340} was found for all ligand concentrations (30−700-fold excess) and pH values (5.3−7.1) employed. The linear dependence between the logarithm of rate constant and the logarithm of ligand concentration is shown in Fig. 2A. The slope of the line is 2.9 ± 0.1 indicating that the reaction is of 3rd order with respect to thiocyanate. The reaction rate increases considerably with decreasing pH values. The dependence of k on pH, at a 200-fold excess of ligand is shown in Fig. 2B. The reaction rate decreases down to half the maximum value at a pH of about 5.6. The fall in A_{340} was accompanied by the increasing intensity of mononuclear Cu^{2+} EPR signal at the same rate.

Fig. 1. Change of oxyhemocyanin absorption at 340 nm during the reaction with a 200-fold excess of thiocyanate, and a 50-fold excess of nitrite, respectively (hemocyanin concentration 0.4 mM with respect to copper, pH 6.0, path length 2 mm, room temperature).
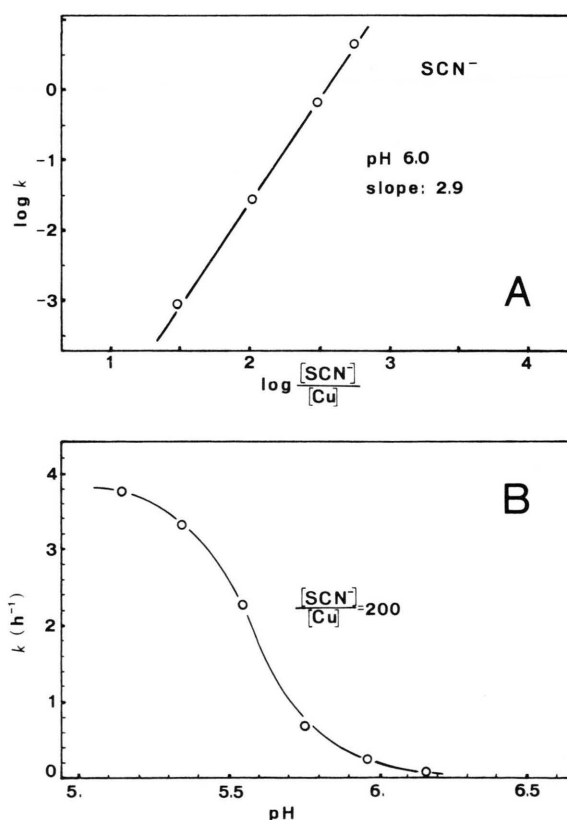

Fig. 2A. Relationship between pseudo first-order rate constant and thiocyanate concentration.
B. Pseudo first-order rate constant as a function of pH.

The decay of A_{340} induced by even much lower concentrations of nitrite is rapid, in comparison with thiocyanate (Fig. 1). Again an exponential decay of A_{340} was observed. Fig. 3A presents the dependence of log k on the logarithm of ligand concentration for two pH values. In this case the reaction was first order also with respect to nitrite (slope 0.95 at pH 5.9 and 1.12 at pH 6.4). Also for this ligand the dependence of the rate constant on pH is similar and its value decreases down to half the maximum rate at a pH of about 5.75 (50-fold ligand excess; Fig. 3B). This concentration of nitrite is sufficient for complete disappearance of the 340 nm band.

Apart from the main effect, i.e. the disappearance of the absorption band at 340 nm, a weak and broad band at 420 nm was observed after thiocyanate treatment. This band corresponds to a CT ligand $\rightarrow Cu^{2+}$ transition, demonstrating a binding of thiocyanate to the active site copper [8].

Fig. 4. EPR spectrum of oxyhemocyanin treated with a 200-fold excess of thiocyanate and with a 50-fold excess of nitrite, respectively, at pH 5.9 (copper concentration in samples 6 mM, microwave frequency 9.28 GHz, modulation amplitude 12.5 Gs, temperature -150 °C).
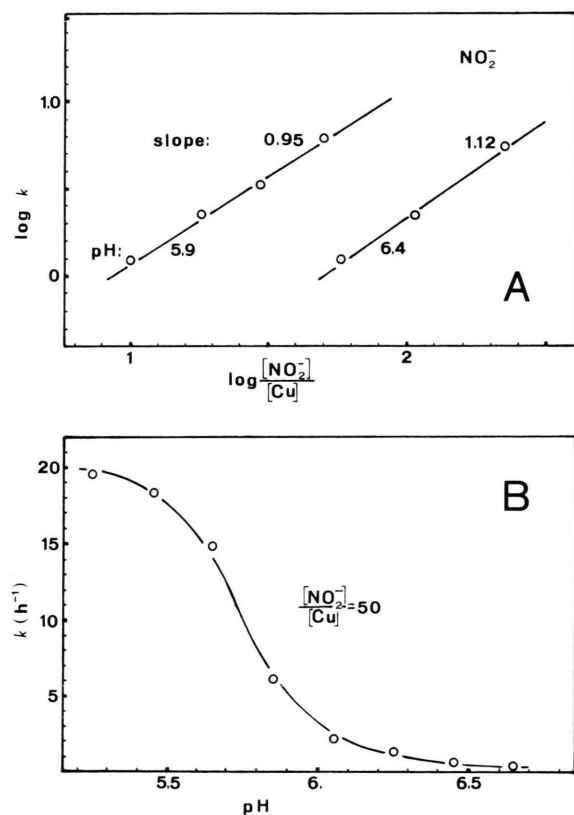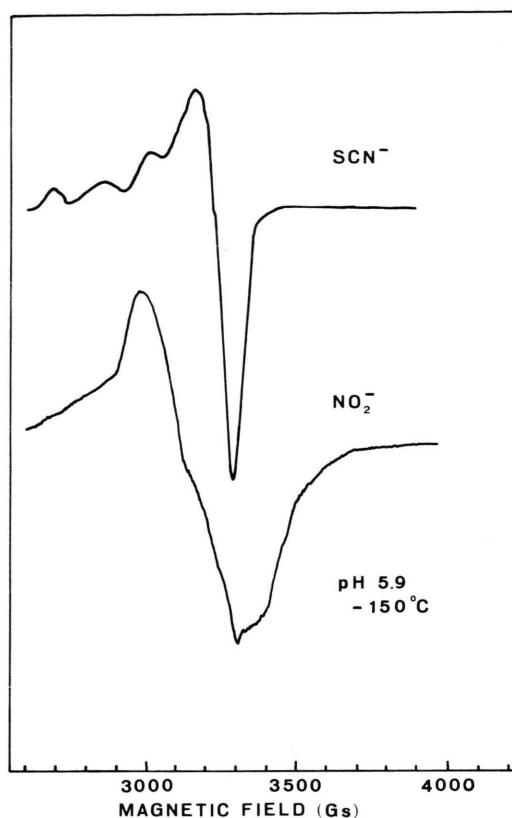

Fig. 3A. Relationship between pseudo first-order rate constant and nitrite concentration at two different pH values. B. Pseudo first-order rate constant as a function of pH.

Changes of magnetic properties of the active site accompanied the alterations in absorption spectra. Treatment of oxyhemocyanin with an excess of SCN^- resulted in an appearance of a mononuclear cupric signal as illustrated in Fig. 4. The following EPR parameters were obtained: $g_{,,} = 2.31$, $g_{\perp} = 2.08$ and $A_{,,} = 155$ Gs. After prolonged incubation with thiocyanate, the amount of copper yielding the EPR signal was estimated at about 40%. A different spectrum was obtained for oxyhemocyanin treated with a 50-fold excess of nitrite as presented in Fig. 4. A characteristic spectrum of a coupled Cu^{2+} ion [9] appeared without a detectable contribution of a mononuclear signal. A reaction of NO_2^- with oxyhemocyanin of *Helix pomatia* demonstrated a mixture of mono- and binuclear signals [6].

Further measurements were performed on solutions dialysed in order to remove the excess of the

ligands added. For thiocyanate ions, a disappearance of the 420 nm band was observed. Hydroxylamine which is capable of reducing mollusc methemocyanin [7], employed in up to 300-fold excess, did not bring about oxyhemocyanin formation both in acidic and in alkaline medium even after one day of incubation. The intensity of EPR spectrum did not change after removing the excess of thiocyanate by dialysis while reduction was observed after removing the excess nitrite by dialysis. A very slow reappearance of oxyhemocyanin was observed during prolonged (500 hours) dialysis at acidic pH of ligand treated oxyhemocyanin against hydroxylamine.

None of the ligands employed induced a weakening of the copper binding to the protein. No decrease in the copper content was found after 24 h dialysis against EDTA.

Discussion

The 340 nm absorption band is of the CT $O_2^{2-} \rightarrow Cu^{2+}$ type [1] and its disappearance indicates a removal of the peroxide anion from the active site. The $SCN^- \rightarrow Cu^{2+}$ band appearing in parallel and disappearing again after dialysis demonstrates a reversible ligand binding to the active site. However, since the typical oxyhemocyanin spectrum does not reappear, the ligand employed does obviously not induce formation of deoxyhemocyanin as it was found for mollusc hemocyanin [5]. At the same time, the EPR signal of mononuclear Cu^{2+} (the intensity corresponding to about 40% of total copper) which appears after termination of the reaction with thiocyanate, suggests a weak binding of the ligand to single Cu^{2+} ions formed due to the disruption of the active sites. This effect was first demonstrated by McMahill and Mason [10] who studied the reaction of *Cancer magister* methemocyanin (generated by H_2O_2) with azide. Our EPR studies of the reaction of *Orconectes limosus* oxyhemocyanin with thiocyanate show the appearance of a mononuclear signal only, without a binuclear copper signal. It seems that thiocyanate ions, after displacing oxygen from the active site, bind to the copper inducing an almost immediate disruption of the active site. After ligand displacement the EPR signal remained indicating that the disruption was permanent.

The results do not differ qualitatively from those reported for hemocyanins of some marine arthropods [8] but are distinctly different from those ob-

tained with molluscan hemocyanins where the content of EPR detectable copper is only a few percent [8]. Other authors, however, have suggested a higher content [11].

The remaining, EPR silent copper occurs in the form of methemocyanin with two Cu^{2+} ions in its active site, which are strongly antiferromagnetically coupled via an internal protein bridge which, as in the case of oxyhemocyanin, accounts for the lack of an EPR signal [12, 13]. Such an explanation is confirmed by the observation of the oxyhemocyanin spectrum reappearing after a prolonged dialysis against hydroxylamine.

The reaction of *Orconectes limosus* oxyhemocyanin with thiocyanate, shows a relatively high order with respect to thiocyanate, about 3 (Fig. 2A). Assuming that the order of a reaction roughly corresponds to its molecularity, one can conclude that the active site of hemocyanin reacts with three molecules of the ligand, which could lead to the following structure:

$$
\begin{array}{ccc}
SCN^- & & SCN^- \\
| & & | \\
(Im) - Cu^{2+} & & Cu^{2+} - (Im) \\
| & & | \\
SCN^- & & R \\
\end{array}
$$
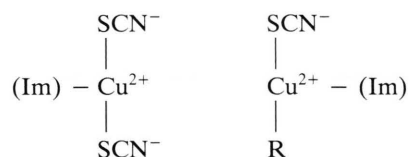
where R is a broken internal protein bridge and (Im) are (two [14] or three [15]) histidine imidazoles to which copper is directly bound. The intercopper distance is long enough to reduce any metal-metal coupling. Such a structure would yield a mononuclear EPR spectrum (Fig. 4).

On the other hand, the reaction with NO_2^- is first-order with respect to nitrite. Therefore if we followed the previous assumption, viz. that the order of reaction corresponds to its molecularity, the active site of hemocyanin would react with one nitrite ion. The characteristic binuclear EPR spectrum suggests the distance between cupric ions to be greater than 5 Å [9] and that an internal protein bridge is broken [10]. The cupric ions are probably bridged by an external ligand to keep the Cu-Cu distance fixed. Due to the complex nitrite chemistry at acidic pH the final form of the ligand bound is hard to settle [6, 8].

From the pH dependence of the reaction rates it can be seen that these values are half-maximum at a pH of about 5.6—5.8. The pK$_a$ values of the ligands employed are: 4.0 for HSCN and 3.4 for HNO$_2$, hence the reaction rates are not determined primari-

ly by the content of the protonated form of the ligand. Lontie [13] reported a pK$_a$ value of 5.9 for the reaction of azide with *Limulus polyphemus* methemocyanin, suggesting that H$^+$ ions play a role as heterotropic effectors in the binding of azide to the active site. The observed pH dependence of the reactions and pK$_a$ value for the reaction of *Limulus polyphemus* methemocyanin are close to the pK$_a$ of histidine (pK$_a$ = 6). The considerable steepness and the shift to lower pH values of the curves in Fig. 2B and Fig. 3B in comparison to the histidine titration curve may be a consequence of independent deprotonation of several imidazole residues in the protein.

An increase in the oxygen affinity with decreasing pH was established for the hemocyanins of *Limulus polyphemus* and some other arthropods [16]. How-ever, a strong correlation between the pH dependence of oxygen affinity and of the reaction rates with other ligands seems unlikely since in the case of *Helix pomatia* hemocyanin a similar pH effect on the reaction rates with ligands has been observed [2] though in this case an increase of oxygen affinity was found with increasing pH [16]. The charge of the ligand therefore seems to play an essential role in this interaction.

Acknowledgements

[1] J. A. Larrabee and T. G. Spiro, J. Am. Chem. Soc. **102**, 4217−4223 (1980).
[2] R. Witters and R. Lontie, FEBS Lett. **60**, 400−403 (1975).
[3] A. J. M. Schoot Uiterkamp, FEBS Lett. **20**, 93−96 (1972).
[4] A. F. Hepp, R. S. Himmelwright, N. C. Eickmann, and E. I. Solomon, Biochem. Biophys. Res. Comm. **89**, 1050−1057 (1979).
[5] W. Rombauts and R. Lontie, Arch. Intern. Physiol. Biochim. **68**, 695−696 (1960).
[6] J. Verpleatse, P. van Tornout, G. Defreyn, R. Witters, and R. Lontie, Eur. J. Biochem. **95**, 327−331 (1979).
[7] H. van der Deen and H. Hoving, Biochemistry **16**, 3519−3525 (1977).
[8] R. S. Himmelwright, N. C. Eickman, C. D. LuBien, and E. I. Solomon, J. Am. Chem. Soc. **102**, 5378−5388 (1980).
[9] A. J. M. Schoot Uiterkamp, H. van der Deen, A. C. J. Berendsen, and J. F. Boas, Biochim. Biophys. Acta **327**, 407−425 (1974).
[10] P. McMachill and H. S. Mason, Biochem. Biophys. Res. Comm. **84**, 749−754 (1978).
[11] R. Witters, M. de Ley, and R. Lontie, Structure and Function of Haemocyanin (J. V. Bannister, ed.), pp. 239−244, Springer-Verlag, Berlin 1977.
[12] N. Makino, H. van der Deen, P. McMahill, D. C. Gould, T. H. Moss, C. Simo, and H. S. Mason, Biochim. Biophys. Acta **532**, 315−326 (1978).
[13] H. R. Lijen, R. Witters, and R. Lontie, FEBS Lett. **88**, 358−360 (1978).
[14] M. S. Co, K. O. Hodson, Th. K. Eccles, and R. Lontie, J. Am. Chem. Soc. **103**, 984−986 (1981).
[15] J. M. Brown, L. Powers, B. Kincaid, J. A. Larrabee, and T. C. Spiro, J. Am. Chem. Soc. **102**, 4210−4216 (1980).
[16] C. P. Mangum, Amer. Zool. **20**, 19−45 (1980).